Qualitative and Quantitative Determination of Caseins with Reverse-Phase and Anion-Exchange HPLC

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ABSTRACT

Whole native caseinate (WNC) and casein (CN) fractions from preparative DE-52 cellulose urea columns were chromatographed using C-8 reverse-phase (RP) and DEAE-type anion-exchange (AEx) HPLC systems. With RP, α_{S2}-CN and κ-CN eluted first as several small peaks; α_{S1}-CN eluted later as two peaks, followed by β-CN peaks. With AEx, κ-CN eluted early as a group of peaks, β-CN eluted next, and α_{S1}-CN and α_{S2}-CN coeluted last. Standard curves were prepared for α_{S1}-CN and β-CN using RP-PHLC and showed correlation coefficients of 0.99 and 0.98, respectively. The caseins in WNC, nonfat dry milk casein, commercial casein(ates) and caseins from milks of individual cows were determined.

INTRODUCTION

THE PHOSPHOPROTEINS that precipitate from raw skim milk at pH 4.6 (caseins) are a heterogeneous mixture of 4 major caseins (CN): α_{S1}-CN, β-CN, α_{S2}-CN, and κ-CN (Eigel et al., 1984). Each of these proteins has unique properties which contribute to the stability of the casein micelle in milk (Farrell, 1988) and to the functionality of casein in dairy products and imitation milk products (Bringe and Kinsella, 1987). Many fractionation schemes have been used to separate the caseins into individual components (Whitney, R. McL., 1988). The most successful method, salt gradient weak anion-exchange chromatography at pH 7 with urea and a reducing agent, was devised by Thompson (1966) and has been used to prepare reference caseins (Creamer et al., 1981; Andrews and Alichanidis, 1983; Humphrey and Newsome, 1984). With this method, fractions consisting of κ-CN, β-CN, α_{S2}-CN, and α_{S1}-CN can be isolated and, after dialysis to remove urea and salts, quantified.

HPLC methods have also been used to separate the caseins. Reverse-phase (RP) C-18 (Visser et al., 1986; Carles, 1986), RP C-4 (Parris et al., 1990), and RP phenyl (Visser et al., 1986) HPLC columns have been used to separate caseins but no data on quantification were reported. Anion-exchange (AEx) DEAE (Visser et al., 1986; Aoki et al., 1987) and AEx quaternary ammonium HPLC columns (Andrews et al., 1985), as well as cation-exchange (Andrews et al., 1985) and hydroxyapatite (Visser et al., 1986) HPLC columns, were used to separate caseins into their major components, but little data on quantification were presented. Size-exclusion HPLC (2000SW (Dimenna and Segall, 1981), 3000SW (Gupta, 1983) and 4000SW (Aoki et al., 1987)) did not separate caseins. Quantification of α_{s1}-CN and β-CN for one whole native casein sample was carried out on AEx DEAE-HPLC by Humphrey and Newsome (1984). Gupta (1983) quantitatively measured total casein but the size-exclusion HPLC system did not separate the caseins.

The objective of our experiments was to develop and evaluate HPLC methods as qualitative techniques for identification of caseins and as quantitative techniques to measure relative quantities of individual caseins, κ-CN, β-CN, α_{S2}-CN, and α_{S1}-CN, in a variety of commercial and experimentally prepared caseins and caseinates. We used DE-52 chromatography to prepare reference samples of the major components and used HPLC methods and SDS and urea PAGE to evaluate purification of κ-CN, β-CN, α_{S2}-CN and α_{S1}-CN by DE-52 chromatography and to identify some minor components and contaminants.

MATERIALS & METHODS

HPLC chromatography

High performance liquid chromatography (HPLC) equipment consisted of a Spectra-Physics 8800 titanium ternary pump equipped with a dynamic mixer, a Rheodyne injection port with a 50-μL sample loop, a Spectra-Physics 8490 UV-Vis detector set at 280 mm and a range of 0.1 AUFS, and a Spectra-Physics 4290 integrator. The chromatographic equipment was controlled through, and the chromatograms were analyzed with, Spectra-Physics ChromStation Autolab software and an Epson Equity II+ computer.

Reverse-Phase HPLC.

A 220 × 4.6 mm reverse-phase C-8 column (Brownlee RP-300 10 μm A2346–030) with a 15 × 3.2 mm guard column of the same material was used for reverse-phase HPLC. Solvent A was 0.1% trifluoroacetic (TFA) in water and solvent B was 0.1% TFA in acetonitrile. The initial mobile phase was 70:30 solvent A: solvent B. Caseins were eluted with a linear gradient to 50:50 A:B in 30 min. After 30 min, a down gradient to 70:30 A:B was run in 15 min with subsequent isocratic flow of 70:30 A:B for 5 min. The down gradient and the isocratic portion of the chromatographic run were necessary to obtain reproducible separations with this column. The flow rate was 1 mL/min. All solvents were HPLC grade. Protein samples for reverse-phase HPLC were prepared in 4.5 M urea in 70:30 A:B with 0.1% mercaptoethanol. All samples were filtered through a 0.45-μm filter before injection. Sample concentration before filtration ranged from 0.7 to 5 mg/mL; 50 μL was injected.

Anion-Exchange HPLC.

A 220 × 4.6 mm weak anion exchange column (Brownlee Aquapore AX-300) with an Anion 15 × 3.2 mm guard column was used for AEx HPLC. Buffer A was 4.5 M Urea in 20 mM TRIS pH 7.0 buffer and Buffer B was the same except that it contained 1M NaCl. The caseins were eluted using the following protocol: isocratic with buffer A for 5 min, a linear NaCl gradient to 0.6M NaCl in 30 min, then to 0.75M NaCl in 10 min, isocratic for 10 min at 0.75M NaCl, a down gradient to buffer A in 20 min, and isocratic in buffer A for 5 min. Total run time was 80 min. Flow rate was 1 mL/min. Protein samples for AEx were prepared in 4.5M Urea, 20 mM TRIS pH 7.0 buffer with 0.1% mercaptoethanol. They were filtered through a 0.45-μm filter before 50 μL was injected. Sample concentration ranged from 0.7 to 5 mg/mL. Buffers were prepared from ultra-pure urea purified before use by passage of 6 M solutions through a 0.45-μm filter and an anion-exchange resin.

DE-52 chromatography

This was carried out as described by Thompson (1966) with the following modifications. DE-52 cellulose was substituted for ordinary DEAE. The column dimensions were 2.5 × 30 cm. The protein load on the column was about 2g. The chromatogram was developed with

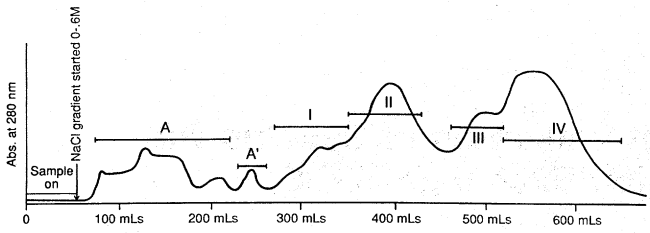

Fig. 1–DE-52 chromatogram of whole native casein from pooled milk.

a gradient of 0 to 0.4M NaCl in 0.01M imidazole, 3.3M urea, 6mM 2-mercaptoethanol pH 7 buffer. The elution pattern was monitored with an ISCO model UA-5 at 280 mm and the fractions were collected every 10 min with a Model 328 ISCO fraction collector. Liquid flow into the column was controlled with a Pharmacia peristaltic pump P-1 and a head of about 1 cm was maintained on the column. Flow rate was about 1.0 mL/min. The fraction numbering scheme is shown in Fig. 1. Fractions were exhaustively dialyzed against water and freeze-dried. Recoveries were estimated from the weights of freeze-dried fractions. Fractions II and IV contained the reference caseins, β-CN and α_{s1}-CN, respectively, which were used for quantification. Fraction I was rechromatographed on DE-52, same condition as above, except that a 0 to 0.2 M linear NaCl gradient was used to obtain purified κ-CN. Fraction III was rechromatographed on DE-52, (same conditions as above), except a 0 to 0.3M linear NaCl gradient was used to obtain purified α_{s2}-CN.

Polyacrylamide gel electrophoresis (PAGE)

Urea-PAGE. Miniature pre-cast 8-25% gradient gels and Pharmacia PhastSystem® were used to obtain PAGE profiles of the fractions. Those gels used for the urea-PAGE analysis were prepared by soaking thegel for 15 min in 6.6M urea in pH 6.4 112 mM TRIS, 112 mM Na acetate buffer. After soaking, gels were used immediately. Native buffer strips were used. Analysis conditions were: 15°C, 300 V, 7.5 mA, 5 W, for a total of 130 Vhr. Staining and destaining were as described in PhastSystem® development technique file no. 200, with the exceptions that staining time was increased to 10 min and destaining was completed by soaking gels (in plastic Petri dishes) in destaining solution for an additional 16 h (overnight) before treating with the preserving solution in the Petri dish. Samples (1 mg) for urea-PAGE were dissolved in 100 μL of the same solution used to soak gels, to which 1 μL 2-mercaptoethanol and 20 μL bromophenol blue (0.02% in 10 mM TRIS, 1 mM EDTA, pH 8) were added.

SDS-PAGE. The 8-25% gradient gels were used as supplied for SDS-PAGE. SDS buffer strips were used and analysis conditions were: 15°C, 250 V, 10 mA, 3 W, for a total of 80 Vhr. Staining and destaining were as described for urea-PAGE. Samples (0.5 mg) were dissolved in 100 μL, 10% SDS, 10 mM TRIS, 1 mM EDTA, pH 8 to which 1 μL 2-mercaptoethanol was added. Samples were heated 5 min at 100°C, centrifuged 10 min in a microfuge and 10μL 0.02% bromophenol blue solution was added.

Protein samples

Whole native caseinate (WNC) was prepared from fresh pooled skim milk by isoelectric precipitation at pH 4.6 with 1N HCl. The casein was washed with distilled water and redissolved, in about the same volume of water as the original milk, by addition of 1N NaOH. The casein was reprecipitated, redissolved at pH 7 and freeze-dried. Caseinate was also prepared in the same manner from reconstituted low-heat-treated dried skim milk (NFDM). Caseinates from milks of individual cows were prepared as described. Genetic typing was according to Kiddy et al. (1964) and Thompson et al. (1964). Purified genetic variants β-CN A^1 and β-CN B were gifts of E. Bingham and H. Farrell. Commercial sodium (Alanate 130), and calcium (Alanate 310) caseinates and rennet (Alanate 771) casein were purchased (New Zealand Milk Products, Inc., Petaluma, CA). Dephosphorylated α_{S1}-CN was prepared as described by Van Hekken et al. (1990). Rennetted κ-CN was prepared by treatment with Hansen's rennet (Chr. Hansen's Laboratories, Milwaukee, WI). Protein recovery for the caseins and caseinates was calculated by normalizing the areas of β-CN to α_{S1}-CN peaks. Total recovery of casein was determined by total normalized peak area using the α_{S1}-CN standard curve.

RESULTS & DISCUSSION

FIGURE 1 shows a typical DE-52 chromatographic analysis of whole native casein accompanied by the SDS (Fig. 2A) and urea-PAGE gels (Fig. 2B). Fraction A contained γ_2 (β-CN (f106–209)) and several minor components which were, possibly, other casein fragments (Fig. 2A and B lane 2). Fraction A′ contained γ_3-CN (β-CN (f108–209)), a slightly smaller fragment of β-CN (Fig. 2A and B, lane 3). Fraction I contained

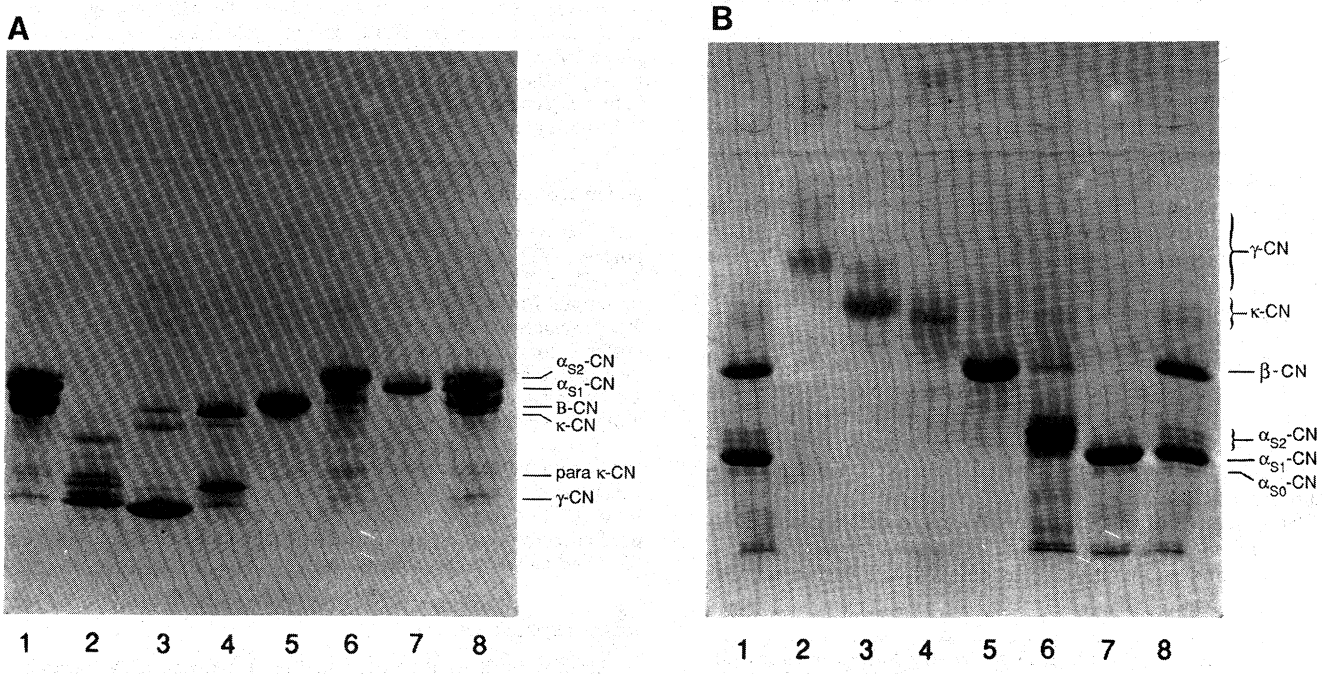

Fig. 2–(A) SDS-PAGE of fractions from DE-52 chromatogram (0 to 0.4M NaC1). (B) Urea-PAGE of fraction from DE-52 chromatogram (0 to 0.4M NaC1): Lane 1 and 8, Whole native casein; Lane 2, Fraction A; Lane 3, Fraction A'; Lane 4, Fraction I; Lane 5, Fraction II; Lane 6, Fraction III; Lane 7, Fraction IV.

Table 1—Absorbances of 1% solutions of various caseins and casein fragments.

Protein	$A^{1\%}_{1cm}$ at 280 nm	Reference
α_{S1}-CN	10.1	Eigel et al. (1984)
α_{S2}-CN	10.7	calculated[a]
κ-CN	10.5	Eigel et al. (1984)
κ-CN Fragments		
para-κ-CN (fl-105)	13.9	calculated
κ-CN (fl06-169)	---	no aromatic amino acids
β-CN	4.6	Eigel et al. (1984)
β-CN Fragments		
β-CN 1P(f29-209) (γ_1-CN)	5.4	calculated
β-CN (f106-209) (γ_2-CN)	8.2	calculated
β-CN (f108-209) (γ_3-CN)	8.3	calculated
β-CN 5P(f1-105)	1.05	calculated
β-CN 5P(f1-107)	1.03	calculated
β-CN 1P(f29-105)	1.54	calculated
β-CN 1P(f29-107)	1.49	calculated
β-CN 4P(f1-28)	---	no aromatic amino acids

[a] Calculated by the method in Cantor and Schimmel (1980) from the primary structure shown in Eigel et al. (1984).

κ-CN, which exists as two genetic variants both of which were glycosylated and unglycosylated, and γ-CN as well as other fragments (Fig. 2A and B, lane 4). Phast urea-PAGE of fractions A, A′ and I were not resolved (Fig. 2B, lanes 2, 3, and 4) (bands were blurred). The Phast SDS-PAGE of these fractions showed better resolution (Fig. 2A, lanes 2, 3, and 4). Fraction II (Fig. 2 lanes 5) contained β-CN and the SDS-PAGE (Fig. 2A, lane 5) showed a pure protein. Urea-PAGE (Fig. 2B, lane 5) showed some minor degradation products of β-CN. Fraction III (Fig. 2, lanes 6) contained α_{S2}-CN and several contaminants, as shown by both urea-PAGE and SDS-PAGE. Fraction IV (Fig. 2, lanes 7) contained α_{S1}-CN. The urea-PAGE indicated some contamination, but the SDS-PAGE showed only α_{S1}-CN and a trace of α_{S2}-CN.

The RP-C8 column was stable throughout the series. However, the AEx column continuously degraded; that is, as the column was used, retention times of caseins decreased. Those caseins (e.g., α_{S1}-CN) with longer retention times showed greatest decreases. Attempts at regenerating the column were unsuccessful. Because of the degradation of the AEx column, only those samples which were run serially could be compared and, when describing results from AEx HPLC chromatography, relative terms were used rather than specific retention times. The response of samples on HPLC (i.e., peak area/mg injection) reflected the absorbance of a 1% protein solution ($A^{1\%}_{1cm}$) at 280 mm and could be used as an aid in identification of specific components. Table 1 lists the $A^{1\%}_{1cm}$ for caseins and β-CN and κ-CN fragments found in WNC preparations.

Figure 3 shows the AEx HPLC and the RP-C8 HPLC of fractions collected from the DE-52 column. On AEx, fraction A (Fig 3A,1) and A′ (Fig 3A,2) eluted in the isocratic portions of the chromatographic analysis immediately after the solvent peak. On RP-C8, fractions A (Fig 3B,1) and A′ (Fig 3B,2) eluted at about 19 min, just ahead of β-CN (Fig 3B,4). The AEx HPLC chromatogram of fraction I (Fig 3B,3) showed a peak eluting immediately after the solvent peak during the isocratic portion of the elution and four major peaks eluting after about 20 min. The RP-C8 HPLC (Fig 3B,3) showed a much more complicated pattern.

Fraction I was rechromatographed on DE-52 cellulose using a lower salt gradient (0 to 0.2 M NaCl) to obtain purer samples of κ-CN (Fig. 4). Eight components were recovered from the rechromatography of Fraction I. Three, which eluted at salt concentrations of <0.03M NaCl (Fraction I-A, I-B, I-C, Fig. 4), migrated on SDS-PAGE in the γ-CN zones. On AEx, fractions I-A, I-B and I-C eluted in the initial isocratic portion of the run as did the γ-CNs. However, on RP-C8 they eluted as two major peaks with retention times (RT) of 7.2 min and 8.5 min and had strong responses (absorbance at 280 mm/mg sample injected) suggesting that they may have been casein fragments other than the γ-CN's, possibly para-κ-CN. The RT of para-κ-CN on the RP-C8 column was confirmed by comparison with a para-κ-CN prepared from κ-CN treated with rennet (Fig. 3B,3).

A series of five components (I-D, I-E, I-F, I-G, and I-H, Fig. 4) eluted from the De-52 0–0.2M NaCl column at salt concentrations of 0.075 to 0.14 M. SDS-PAGE of component I-D showed mostly κ-CN and traces of γ_3-CN. On AEx-HPLC, component I-D had 2 major peaks on AEx-HPLC in the κ-CN region (Fig. 3A,3), while the RP-C8 HPLC showed 2 small peaks at RT's of 10 and 11 min and one large peak at 17 min (Fig. 3B,3). In contrast to the SDS-PAGE results, RP-C8 HPLC results indicated that this component was mostly γ-CN with about 20% κ-CN. Components I-E and I-F were pure κ-CN by both SDS-PAGE and urea-PAGE. On AEx-HPLC (Fig. 3A), both component I-E and I-F split into several peaks which may have been due to genetic variants and/or differences in glycosylation of the κ-CN. On RP-C8 HPLC component I-E showed a single peak with a RT of 10.5 and component I-F eluted as four peaks with RT of 9.3 min, a broad peak with RT of 13.7 min with sharp peaks at RT's of 11.5 and 12.6 min (Fig. 3B,3). Component I-G (Fig. 4) contained κ-CN and 2 lower-molecular-weight fragments as shown by SDS-PAGE. On AEx, peak I-G had a large peak eluting in the isocratic portion of the run as well as κ-CN peaks. On RP-C8, component I-G eluted as κ-CN plus para-κ-CN. Seventy-five percent of component I-G was κ-CN, by RP-C8 HPLC. Component 1-H (Fig. 4) contained (by SDS-PAGE) lower-molecular-weight fragments only.

HPLC provided rapid analysis of fraction I and information on amount and possible composition of contaminants. The major peaks for κ-CN in RP-C8 were those with RT's between 9 and 13.5 min. The major contaminants eluted either earlier or later than the κ-CN peaks. The major peaks for κ-CN in AEx were 5 peaks with different RT when the κ-CN peaks from the DE-52 (0 to 0.2 M NaCl) were run separately. When fraction I was analyzed, only 4 peaks were present in the κ-CN region because the last two coeluted. The major contaminants eluted earlier than κ-CN.

Fraction II contained β-CN by both urea-PAGE and SDS-PAGE. The AEx chromatogram (Fig 3A,4) showed a single sharp peak with a RT intermediate between fraction I and fraction IV (α_{S1}-CN). The RP-C8 (Fig 3B,4) chromatograms showed a double peak for β-CN. The double peak for β-CN could be due to self-association of the β-CN. However, the chromatographic conditions, except temperature, did not tend to promote self-associations (Payens et al., 1969). β-CNs are also known to separate on a RP-C18 column on the basis of genetic variation in uncharged amino acids (Carles, 1986). Samples of known genetic composition were chromatographed on RP-C8; β-CN A^1 and two β-CN B's were injected. One β-CN B showed a single peak at 20.3 min while the other β-CN B (from another animal) showed two peaks, one at 20.5 and one at 22 min. The β-CN A^1 also showed two peaks, one at 21.2 and another at 22.6 min. WNC's from cows typed as β-CN A or B were also injected and the number of β-CN peaks varied between animals. Milk typed as containing β-CN A (whether it contained A^1, A^2, or A^3 was not determined) gave four β-CN peaks. Milks typed as β-CN A,B gave 2 peaks in one case and 5 in another. These results suggested that RP-C8 HPLC could separate β-CNs on the basis of known genetic differences and that self-association of the β-CN occurred on the C-8 column, but not for all variants of β-CN. The double peak found for β-CN in pooled milk was the result of both complex associations and genetic variation.

Fraction III contained α_{S2}-CN as well as several contaminants as shown by SDS-PAGE and urea-PAGE. Fraction III (Fig. 3A and B, 5) also showed complex chromatograms on both AEx and RP-C8. Pure samples of α_{S2}-CN were prepared by rechromatography on DE-52 using a 0–0.3 M NaCl gradient (Fig. 5). Five components eluted from the DE-52 0–0.3M NaCl column, labeled III-A, III-B, III-C, III-D, and III-E. Com-

Table 2—Quantification of individual caseins—DE-52 cellulose chromatography and HPLC

Method	Total recovered % (s.d.)	Percent of recovered protein: A % (s.d.)	A′ % (s.d.)	κ-CN % (s.d.)	α_{S2}-CN % (s.d.)	β-CN % (s.d.)	α_{S1}-CN % (s.d.)
DE-52 N=5	75.6[a] (6.3)	3.4 (0.3)	0.8 (0.3)	11.4[a] (2.17)	13.0 (1.5)	37.3[a] (4.3)	34.7[a] (4.1)
Anion Exchange HPLC N=8	72.4[a] (10.5)			16.6[a] (4.3)		27.6[b] (1.7)	47.9 (7.3)[c]
C-8 Reverse Phase HPLC N=11	98.5[b] (9.3)			17.2 (2.5)[d]		37.7[a] (6.2)	39.8[b] (2.2)

[a,b] Means within a column with same superscript do not differ (Student's t, $p>0.05$).
[c] Includes α_{S2}-CN. Retention times coincide.
[d] Includes α_{S2}-CN. Each casein generates several peaks with similar retention times.

Table 3—Total and individual casein recoveries using RP-C8 HPLC.

Sample	Total recovered % (s.d.)	κ-CN and α_{S2}-CN % (s.d.)	β-CN % (s.d.)	α_{S1}-CN % (s.d.)	N
Sodium caseinate	94.8 (13.7)	14.7 (1.6)	45.4 (1.4)	40.8 (1.8)	3
Calcium caseinate	76.4* (8.7)	13.8 (4.3)	42.7* (2.1)	44.9 (5.3)	3
Rennet casein	66.4	4.3	49.7	45.1	1
NFDM caseinate[a]	92.6* (2.6)	12.6* (4.2)	43.2* (2.3)	46.5* (3.2)	6
Individual[b]					
	116	7.8	42.6	35.5	
	111	18.3	46.2	37.0	
	108	16.2	40.1	37.7	
	109	15.7	39.4	41.5	
	115	14.9	42.7	38.5	
	108	13.3	36.7	47.8	
	81.4	20.1	35.1	49.2	

[a] Caseinate made from reconstituted low-heat nonfat dry milk powder.
[b] Caseinates from milks of individual cows.
* Differs significantly from WNC (Table 2). Student's t, $p<0.05$.

separation techniques designed to alter the functionality of this important food ingredient. Further research is needed to confirm the ability of C-8 RP HPLC to distinguish between genetic variants of β-CN and to elucidate the structural and functional reasons why both α_{S1}-CN and β-CN split on the reverse-phase column.

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Ms received 10/3/90; revised 3/2/91; accepted 5/4/91.

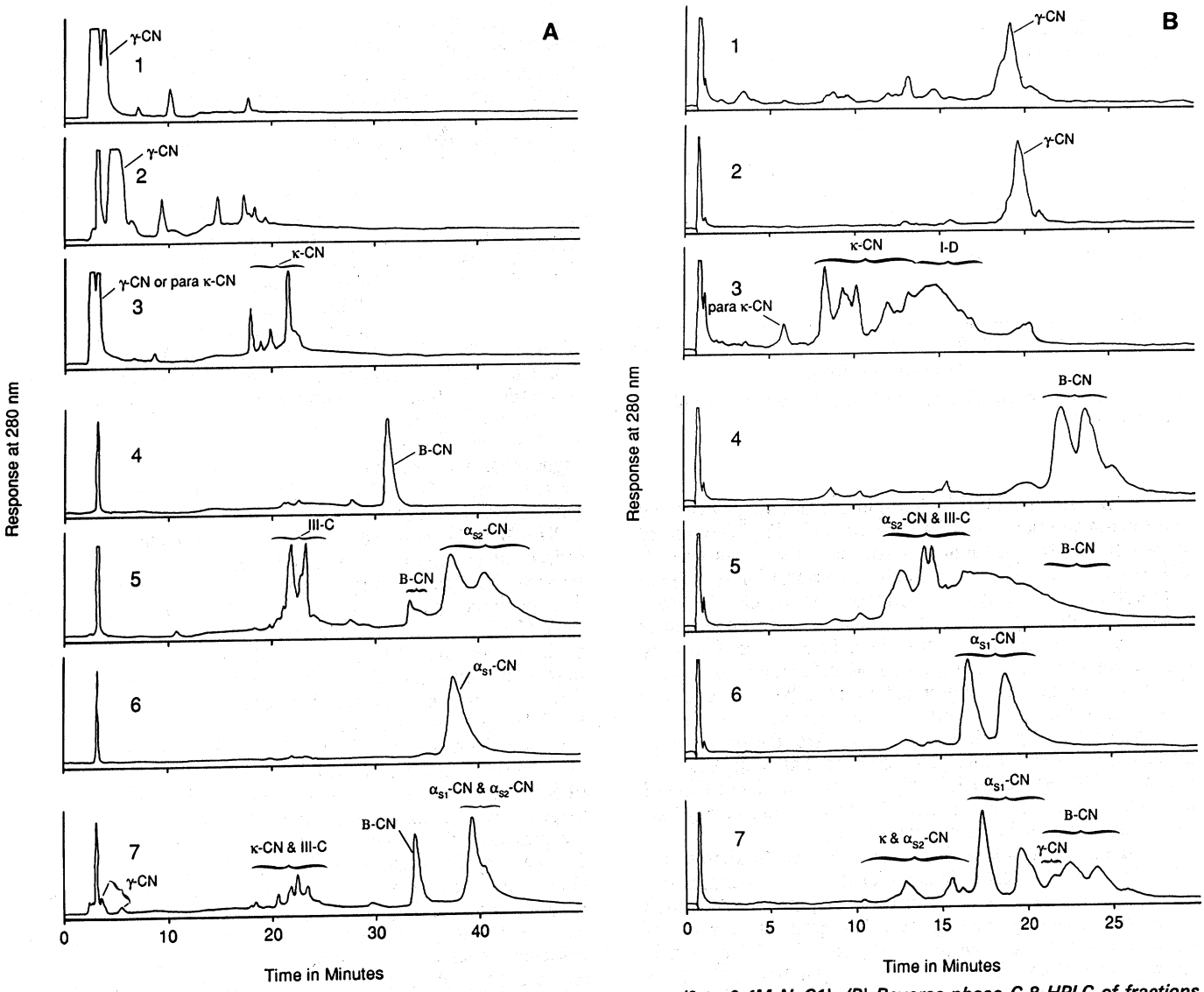

Fig. 3–(A) Anion exchange HPLC of fractions from DE-52 chromatogram (0 to 0.4M NaC1). (B) Reverse-phase C-8 HPLC of fractions from DE-52 chromatogram (0 to 0.4M NaC1): 1. Fraction A; 2. Fraction A'; 3. Fraction I; 4. Fraction II; 5. Fraction III; 6. Fraction IV; 7. Whole native casein.

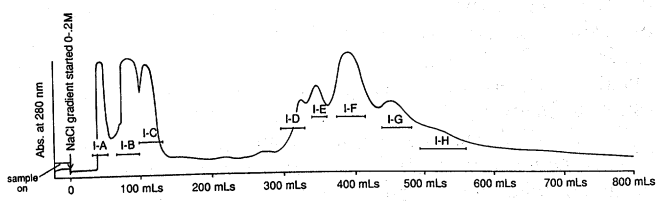

Fig. 4—DE-52 chromatogram of fraction I.

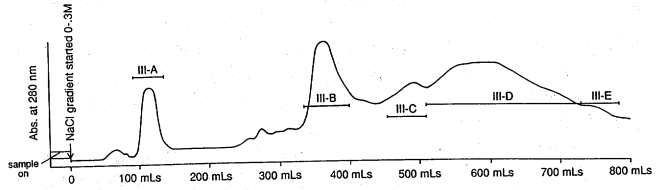

Fig. 5—DE-52 chromatogram of fraction III.

ponent III-A, which eluted at <0.04 M NaCl, yielded no recoverable weight. No further analysis was attempted. Component III-B, which eluted between 0.12 and 0.14 M NaCl, contained β-CN, confirmed by urea and SDS-PAGE. Component III-C eluted between 0.16 and 0.18M NaCl. SDS-PAGE showed a complex pattern with the largest (visually) portion having smaller MW than caseins, a band in the casein area and a band with a larger MW than casein. Urea-PAGE of peak III-C showed the major band moving slightly slower than α_{S2}-CN and two bands which moved faster than α_{S1}-CN, traces of κ-CN and β-CN were also evident. On AEx-HPLC, component III-C eluted about the same time as the first two peaks on the AEx HPLC (Fig. 3A, 5.) of Fraction III. On RP-C8, component III-C eluted as a series of 5 sharp peaks between 12 and 16 min RT. Components III-D and III-E, which eluted between 0.18 and 0.3M NaCl, consisted of α_{S2}-CN and traces of κ-CN and β-CN, as shown by urea- and SDS-PAGE. They eluted from AEx in the same area as the last two peaks in Fig. 3A,5. On RP-C8, they eluted as three peaks, one at 12 min and a doublet at 15.3 and 15.7 min.

On AEx, the analysis of α_{S2}-CN was complicated because α_{S1}-CN and α_{S2}-CN coeluted and the contaminants in component III-C coeluted with κ-CN. However, component III-C would contribute < 10% to the κ-CN quantification. On RP-C8, the analysis of α_{S2}-CN in WNC was complicated because κ-CN and component III-C had about the same RTs, but would cause <10% error in quantification of κ-CN and α_{S2}-CN.

Fraction IV contained α_{S1}-CN by urea- and SDS-PAGE. On

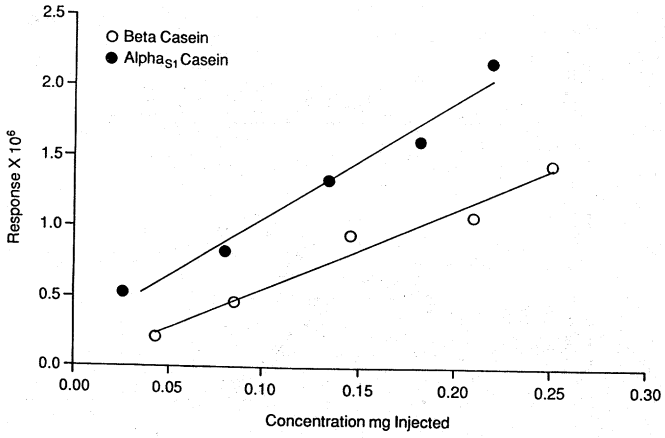

Fig. 6—Response of RP-C8 HPLC versus amounts of α_{S1}-CN and β-CN injected.

AEx-HPLC, fraction IV (Fig 3A,6) eluted as a single peak late in the chromatogram. On RP-C8, fraction IV (Fig 3B,6) eluted as two peaks: 16.5–17 min and 18.5 to 19 min. As with β-CN, α_{S1}-CN can self-associate and this association is concentration-dependent. A series of dilutions of α_{S1}-CN was analyzed. The ratio between the two peaks remained constant at all dilutions. The possibility that the split could be caused by genetic variants was also examined. Milks that contained α_{S1}-CN B showed the same elution pattern as milks that contained α_{S1}-CN C. Another possibility was that because α_{S1}-CN exists with both 8 and 9 protein-bound phosphates, the protein separated on the basis of phosphate content. Partly dephosphorylated α_{S1}-CN was prepared. This protein contained an additional peak but with a slightly shorter RT than the native α_{S1}-CN. The cause of this split peak is unknown.

AEx-HPLC of whole native casein (Fig. 3A,7) showed the following sets of peaks. The small peaks in the isocratic portion of the run were γ-CN and other fragments. The small peaks immediately preceding the κ-CN group were also CN fragments. A group of four peaks were κ-CN's. A large single sharp peak eluting after the κ-CN was β-CN and the large peak eluting after the β-CN was a combination of both α_{S1}-CN and α_{S2}-CN.

RP-C8 of whole native casein (Fig. 3B,7) showed the following peaks. A series of very small peaks which eluted before 5 min was unidentified (less than 1% of the total response). The next series of peaks which elute between 8.5 and 15 min was para-κ-CN, κ-CN and α_{S2}-CN. After κ-CN and α_{S2}-CN eluted, α_{S1}-CN eluted as twin peaks at RT's between 16 and 19 min. γ-Caseins eluted between the α_{S1}-CN and β-CN. β-CN eluted between 19 and 24 min.

To quantify the responses of the HPLC, standard curves (Fig. 6) were prepared for α_{S1}-CN and β-CN on the RP-C8 column. The correlation coefficient for α_{S1}-CN area versus concentration of protein solution before filtration was 0.99 and for β-CN was 0.98. The area under the β-CN peaks was normalized to a α_{S1}-CN area; total area was calculated and the α_{S1}-CN response was used to determine the % total recovery from the amount of protein injected. Total recoveries from DE-52 chromatography, AEx and RP-C8 HPLC are shown in Table 2. The recovery from the DE-52 column was less than that reported by Thompson (1966), but we measured only the material eluting as peaks and no attempt was made to collect all eluted material. The percentages of individual proteins were determined by weight of freeze-dried dialyzed fractions, which were identified as the major casein component. Fractions I and III were about two thirds κ-Cn and α_{S2}-CN, respectively, by rechromatography.

Aex HPLC had percent total recovery not significantly different (Student's t, $p>0.05$) from DE-52 chromatography. There was no significant difference ($p>0.05$) between the percent κ-CN found with AEx HPLC and that found with DE-52 chromatography. The percent α_{S1}-CN and α_{S2}-CN found with AEx (47.9%) was near that found with DE-52 chromatography for α_{S1}-CN and α_{S2}-CN combined (47.7%). However, the percent β-CN found with AEx HPLC was significantly less ($p<0.05$) than found with DE-52 chromatography.

Total recovery from RP-C8 HPLC was significantly higher ($p<0.05$) than recoveries from either AEx HPLC or DE-52 chromatography. Total recoveries for some samples exceeded 100% because both α_{S2}-CN and κ-CN had a higher $A^{1\%}_{1cm}$ than α_{S1}-CN, and some β-CN fragments (γ-CN's) had higher $A^{1\%}_{1cm}$ than did β-CN (Table 1). The percent α_{S2}-CN and κ-CN (17.4%) found with RP-C8 was about 70% of the combined α_{S2}-CN and κ-CN (24.4%) found with DE-52. With RP-C8, the percent β-CN found was not significantly different ($p>0.05$) from that found with DE-52 chromatography but was significantly different ($p<0.05$) from AEx HPLC. The percent α_{S1}-CN measured by RP-C8 HPLC was significantly ($p<0.05$) higher than was measured by DE-52 chromatography. However, the ratio of α_{S1}-CN to β-CN found using RP-C8 agreed with reported ratios (Eigel et al., 1984).

RP-C8 HPLC was used to determine the relative amount of individual caseins in commercial preparations (Table 3). In general, the total recovery of protein from commercial caseins was low in comparison with that made from fresh pooled skim milk (Table 2), but the commercial caseins are not pure. For sodium caseinate, the percent β-CN fond was significantly higher ($p<0.05$) than for WNC (Table 2), and the total percentage recovered as identified caseins (100.9) was higher than for WNC (94.7%). These increases may have been due to the presence of γ-CN (Fig. 3B,7) in WNC which was not included in identified proteins. For calcium caseinate, the percentages of β-CN and α_{S1}-CN were significantly higher ($p<0.05$) than those for WNC. Both α_{S1}-CN and β-CN are more insoluble in the presence of calcium than is κ-CN and this solubility difference may be reflected in the α_{S1}-CN and β-CN content. One sample of rennet casein was analyzed. Values obtained, very low κ-CN and α_{S2}-CN and high β-CN and α_{S1}-CN, were consistent with treatment that destroyed κ-CN. However, no evidence of para-κ-CN was seen in the chromatogram. Caseinate made from reconstituted low-heat-treated NFDM differed significantly ($p<0.05$) from WNC for all parameters. While these differences were statistically significant, they were similar to those found for commercial caseinates. The caseinates prepared from milks of individual cows showed a variety of individual casein contents, ranging from κ-CN and α_{S2}-CN of 7.8% to 20.1%; β-CN of 35.1% to 46.2%, and α_{S1}-CN of 35.5% to 49.2%. When the data from all milks were combined, means for individual percent caseins were not significantly different ($p>0.05$) from means for WNC.

RP-C8 HPLC showed distinct advantages over AEx HPLC. RP-C8 showed almost 100% recovery of protein, reagents used in the chromatographic procedures were easily made, and the column was stable and yielded reproducible results. AEx, in contrast, showed about 75% recovery, buffers used in the chromatographic procedure required extensive clean up, and retention times for the proteins changed dramatically as the column was used. RP-C8 was a useful method for monitoring DE-52 chromatographic purification of reference caseins and was shown to be a simple and effective method for analysis of caseins and caseinates from a variety of sources.

CONCLUSION

C-8 RP HPLC can be used to separate and quantify α_{S1}-CN, β-CN, α_{S2}-CN, and κ-CN, as well as certain casein fragments, γ-CN's (β-CN fragments) and para-κ-CN (a κ-CN fragment), in a variety of commercial and laboratory casein products. Compositional information on casein can be obtained rapidly with RP-HPLC and it will be useful in the rapid evaluation of